TOXICOLOGY AND APPLIED PHARMACOLOGY 141, 416–424 (1996)
ARTICLE NO. 0307

4-Hydroxy-2-nonenal–Protein Adducts and Apoptosis in Murine Lung Cells after Acute Ozone Exposure

ALEXANDER KIRICHENKO,*,† LI LI,* MARIA T. MORANDI,‡ AND ANDRIJ HOLIAN*

*Division of Pulmonary and Critical Care Medicine, Department of Internal Medicine and Pharmacology, Toxicology Program, Medical School, and ‡School of Public Health, University of Texas Houston Health Science Center, Houston, Texas 77030; and †Oncology and Radiology Research Institute, Kyiv, Ukraine

Received December 18, 1995; accepted July 31, 1996

4-Hydroxy-2-nonenal–Protein Adducts and Apoptosis in Murine Lung Cells after Acute Ozone Exposure. KIRICHENKO, A., LI, L., MORANDI, M. T., AND HOLIAN, A. (1996). *Toxicol. Appl. Pharmacol.* 141, 416–424.

Ozone is a photochemically generated pollutant that can cause acute pulmonary inflammation and induce cellular injury and may contribute to the development or exacerbation of chronic lung diseases. Despite extensive investigation, the mechanisms of ozone and oxidant-induced cellular injury are still uncertain. Ozone has been reported to cause the formation of aldehydes in biological fluids that could explain many of the cellular effects caused by ozone. One of the most toxic aldehydes formed during oxidant-induced lipid peroxidation is 4-hydroxy-2-nonenal (HNE). HNE reacts primarily with Cys and secondarily with Lys and His amino acids, altering protein function and forming protein adducts that can be detected using specific adducts. In this study, we investigated whether ozone could cause the formation of HNE by assaying for HNE–protein adducts in cells isolated by lung lavage from C3H/HeJ mice exposed to 2.0 and 0.25 ppm ozone for 3 hr. Since oxidative stress and HNE have been shown to cause apoptosis we also examined the lung lavage cells for evidence of apoptosis following ozone exposure. Using a specific polyclonal antibody against HNE–amino acid adducts, two principle HNE–protein adducts were detected by Western analysis in cells obtained after ozone exposure at approximately 86–90 and 32 kDa. In addition to cell necrosis, apoptosis of lung cells was significant 3 hr after ozone exposure as detected using a Cell Death ELISA procedure and confirmed with DNA ladder and morphological analysis. The apoptotic cell injury peaked at 6 hr postexposure and decreased by 24 hr. Taken together, these results demonstrate that HNE is formed *in vivo* following ozone exposure and HNE appears to form specific protein adducts in lung cells. Furthermore, ozone can cause lung cell injury by an apoptotic mechanism in addition to a necrotic mechanism. Since HNE is toxic to cells and is formed as a result of ozone exposure, it may contribute to the lung cell injury following ozone exposure. © 1996 Academic Press, Inc.

Ozone is one of the most reactive pollutants to which humans are routinely exposed. The functional, biochemical, and cellular effects of ozone exposure in humans, animal models, and *in vitro* systems have been reviewed (Devlin *et al.*, 1991; Wright *et al.*, 1990). A number of studies have demonstrated that acute exposure to ozone produces inflammatory responses and cell damage in the epithelium of the nasal cavity (Bhalla *et al.*, 1986; Harkema *et al.*, 1987), bronchi (Wilson *et al.*, 1984), and small conductive airways, particularly in the terminal airways near the gas exchange region of the lung (Chang *et al.*, 1992; Koren *et al.*, 1989). *In vivo* ozone exposure has also been shown to affect the number and morphology of alveolar macrophages (AM) (Driscoll *et al.*, 1987; Gilmour *et al.*, 1991; Hotchkiss *et al.*, 1989; Pendino *et al.*, 1994; Victor, 1992), and decrease their capability to defend the alveoli and distal airways against inhaled particles and pathogens (Gilmour *et al.*, 1993b; Gilmour and Selgrade, 1993; Jakab and Hemenway, 1994; Loveren *et al.*, 1988; Polzer *et al.*, 1994). In addition to its acute effects, chronic ozone exposure may contribute to significant long-term pulmonary changes, such as interstitial fibrosis (Last *et al.*, 1993; Reiser *et al.*, 1987; Vincent *et al.*, 1989). Although all these effects are well defined, the molecular mechanisms involved in acute oxidant lung injury remain unclear.

Pryor (1986) proposed that ozone injury may be mediated in part by aldehydes that are formed by ozone-induced peroxidation of polyunsaturated fatty acids (PUFA) in lung lining fluid (LLF) and lipophilic regions of the cells. Consistent with this suggestion, it has been shown from *in vitro* studies that aldehydes are reaction products when PUFA from LLF undergo ozonation (Pryor and Church, 1991; Pryor *et al.*, 1991b). Although a large number of aldehydes can be formed during PUFA peroxidation, three aldehydes have been extensively investigated as physiologically relevant lipid peroxidation products (4-hydroxy-2-nonenal (HNE), 4-hydroxy-2-hexenal, and malonaldehyde) (Esterbauer *et al.*, 1991). Among these aldehydes, HNE is a major metabolite and the most toxic product of lipid peroxidation that has been identified (Esterbauer *et al.*, 1986). HNE originates from peroxidation of arachidonic and linolenic acids contained in

0041-008X/96 $18.00
Copyright © 1996 by Academic Press, Inc.
All rights of reproduction in any form reserved.

PM3006739318

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

the polar phospholipids and may be a reliable indicator of free radical-induced lipid peroxidation (Esterbauer *et al.*, 1991). HNE has been shown to be cytotoxic in a dose-dependent manner to a broad spectrum of cells and has been implicated in numerous forms of cell injury (Esterbauer *et al.*, 1991; Zollner *et al.*, 1991). HNE concentrations below 0.1 μM have been shown to stimulate neutrophil chemotaxis (Curzio *et al.*, 1990; Schaur *et al.*, 1991), modulate adenylate and guanylate cyclase activity (Dianzani *et al.*, 1989), and stimulate phospholipases (Rossi *et al.*, 1990; Taher, 1993). Doses of HNE in the 1 to 100 μM range cause different cytotoxic effects including genotoxic and carcinogenic effects and cause cell death (Michiels and Remacle, 1991; VanWinkle *et al.*, 1994). Recently, Li *et al.* (1996) demonstrated that HNE induced apoptosis in murine alveolar macrophages *in vitro* at 25–100 μM, but at higher concentrations HNE induced necrosis of alveolar macrophages. It has been proposed that HNE exerts these effects because of its high reactivity with biological molecules, preferentially with thiol compounds (GSH, cysteine) and proteins containing thiol groups to form thioether adducts that undergo cyclization to form hemiacetals (Danielson *et al.*, 1987). HNE also reacts with other amino acid residues of proteins to form stable Michael-type adducts (Szweda *et al.*, 1993; Uchida and Stadtman, 1993).

Recently, Uchida *et al.* (1993), using a polyclonal antibody against HNE-modified proteins, demonstrated the formation of HNE-modified proteins in oxidized hepatocytes. The formation of HNE–protein adducts has also been shown in the renal proximal tubules of rats treated with the renal carcinogen ferric nitrilotriacetate (Toyokuni *et al.*, 1994). In the present study, we examined HNE–protein adduct formation in lung lavage cells following *in vivo* ozone exposure, in order to estimate the relative importance of HNE in acute ozone injury. Furthermore, since oxidative stress has been implicated as a causative agent for cell apoptosis (Buttke and Sandstrom, 1994) and HNE has been shown to cause apoptosis (Li *et al.*, 1996), we investigated lung lavage cells for apoptosis following ozone exposures.

MATERIALS AND METHODS

Animals. Male C3H/HeJ mice (Jackson Laboratories, Bar Harbor, ME), 8–9 weeks old (approximately 20 g), were used in this study. Mice were housed in microisolators for at least 3 days to acclimate prior to exposure studies and given a standard dry laboratory diet.

Exposure. Mice were exposed for 3 hr to 2.0 and 0.25 (±5%) ppm ozone using 0.75-m^3 portable Laskin-type stainless steel chambers (Wahman, MD). Ozone was produced by passing pure medical-grade oxygen through an electrical ozone generator (OREC Model 03VI-O ozonator, Phoenix, AZ) and injected directly into the air stream just prior to entrance into the exposure chamber. The ozone chamber was operated at negative pressure with respect to ambient conditions. The chamber air exchange rate was approximately 20/hr. The flow of ozone into the chamber was controlled by a flowmeter. The concentration of ozone in the chamber was monitored constantly using a calibrated ultraviolet monitor (Model 1003, Dasibi Environmental Corp., Glendale, CA) and automatically recorded with a strip-chart recorder. Teflon tubing was used to prevent loss of ozone. The calibration of the ozone analyzer was verified at the EPA regional laboratory in Houston, Texas.

For each exposure 18 mice were used. Three served as the control group. Groups of 3 mice were sacrificed immediately after exposure or at 1.5, 3, 6, and 24 hr following exposure. The cells from each group of 3 mice were pooled and served as one sample ($n = 1$). The entire exposure protocol was repeated three times. Therefore, each observation represents $n = 3$, where each n is a pool of cells from 3 mice. For the studies of DNA ladder formation 8 control and 8 exposed mice were euthanized at one time point postexposure; cells from 8 mice were pooled for both the air and ozone groups.

Bronchoalveolar lavage and sample preparation. Mice were anesthetized with 50 mg/kg sodium pentobarbital and exsanguinated by severing the dorsal aorta. Lungs were removed and lavaged 7 times with 0.8 ml of phosphate-buffered saline (PBS) solution (pH 7.2) and collected on ice. Total lung cells were recovered by centrifuging the lavage fluid at 500*g* for 10 min at 4°C. Cells were then resuspended in PBS and counted with a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL). Cell viability was determined by trypan blue exclusion and was routinely greater than 95% in control mice. At least 200 cells were counted for each group. Cells were then cytocentrifuged onto glass slides (3×10^3 cells/slide) for differential staining with Diff-Quick (Fisher Diagnostics, Pittsburgh, PA) or fixed in cold absolute methanol for Wright Giemsa staining (Curtin Matheson Scientific, Inc., Houston, TX) and stored at 4°C. The remaining cells were centrifuged and solubilized in Laemmli sample buffer (2% SDS, 10% glycerol, 60 mM Tris-HCl, pH 6.8, 0.1 M DTT, 0.01% bromphenol blue) at 40 μl/10^6 cells, and samples were sonicated with an ultrasonifier cell disrupter (Model W-225, Heat Systems, Ultrasonic Inc., New York, NY) at room temperature for 30 sec and heated in boiling water for 5 min prior to storage at −80°C.

DNA fragmentation assays. For DNA ladder formation, genomic DNA was isolated from the lavage cells by using the DNA ISOLATOR (Genosys, Woodlands, TX) according to the manufacture's protocol. The isolated genomic DNA was dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and 3′-end-labeled with [α-^{32}P]dCTP (ICN Pharmaceutical, Costa Mesa, CA) by incubation of 1 μg of DNA in 50 μl reaction buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 200 μM dATP, dGTP, dTTP, 2 μl of [α-^{32}P]dCTP, 2 U of klenow) at 37°C for 30 min. The [α-^{32}P]dCTP-labeled DNA was mixed with 10 μl of loading buffer (0.25% bromophenol blue, 100 mM EDTA, 30% glycerol). Identical amounts of [α-^{32}P]dCTP-labeled DNA (50 ng) were loaded onto a 2% agarose gel and run at 5 V/cm for 5 hr in 1× TAE buffer (40 mM Tris-acetate, pH 8.0, with 1 mM EDTA). The gel was dried at 60°C under vacuum in a gel drier and then exposed to X-ray film for detection of labeled DNA fragments.

Cells were also processed and analyzed for apoptosis by the detection of the cytosolic histone-bound DNA fragments using the Cell Death Detection ELISA kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol. The assay is based on the quantitative sandwich-enzyme-immunoassay principle using monoclonal antibodies directed against DNA and histone; 1×10^5 cells from each sample were processed and 5000 cells were used for each reaction. Results are reported as the average of triplicate assays performed for each sample.

SDS–PAGE and Western analysis. Equal amounts of protein were loaded on sodium dodecyl sulfate (SDS)–polyacrylamide minigels (Bio-Rad). After electrophoresis for 45 min at 180 mA the protein was transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL) using a Trans-Blot Electrophoretic Transfer Cell (Bio-Rad). After protein transfer, the nitrocellulose membrane was placed in blocking buffer [5% Blotto in

PM3006739319

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

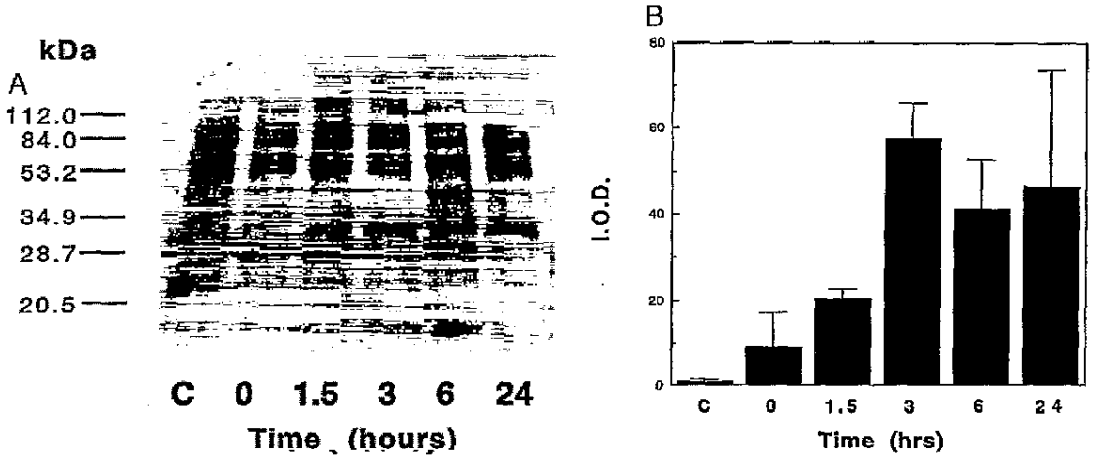

FIG. 1. HNE–protein adducts in murine lung cells following *in vivo* exposure to 2.0 ppm ozone for 3 hr. HNE adducts were detected in lavaged lung cells by Western analysis as described under Materials and Methods. (A) Representative Western of HNE adducts following ozone exposure. Each lane contains pooled cells from three C3H/HeJ mice. Lane 1 represents controls at 3 hr following air exposure. Lanes 2–6 represent lung cells at 0, 1.5, 3, 6, and 24 hr following ozone exposure, respectively. (B) The 32-kDa region of three separate Westerns was scanned and the densitometry results are presented as mean ± SEM of the integrated optical density (I.O.D.). The results show an increase in HNE adducts following ozone exposure.

TNA buffer (10 mM Tris-HCl, 150 mM NaCl, pH 7.2)] for 16 hr at 4°C. After blocking, the membrane was incubated with primary antibody at 1:500 dilution in 15 ml of 5% Blotto in TNA buffer for 16 hr at 4°C. The membrane was washed extensively with TBST (10 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20, pH 8.0) for 2 hr and incubated with secondary antibody at 1:15,000 dilution in TBST for 1.5 hr at room temperature. After washing for 1.5 hr with TBST, the membrane was exposed to enhanced chemiluminescence reagents (ECL) for 1 min followed by autoradiography (Hyperfilm ECL, Amersham) to visualize immunoblot signals. Relative densities of immunoblots were measured with a Shimadzu 9000 densitometer.

Materials. The HNE-specific polyclonal antibody against rabbit HNE–keyhole limpet hemocyanin conjugate was kindly provided by Dr. Luke Szweda (Case Western Reserve University, Cleveland, OH) and is the same antibody described previously (Uchida *et al.*, 1993; Szweda *et al.*, 1993). Donkey anti-rabbit IgG conjugated to horseradish peroxidase secondary antibody was obtained from Amersham. HNE was obtained from Biomol (Plymouth Meeting, PA). Sources of all other chemicals and reagents were noted above.

Statistical analysis. Statistical differences between exposure groups were determined by a one-way analysis of variance (ANOVA) followed by Student Neuman–Keuls tests. Values are reported as the mean ± SEM. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Ozone-Induced HNE–Protein Adducts

The first experiments were to determine whether *in vivo* ozone exposure caused the formation of HNE-modified proteins in lung cells. As can be seen from Fig. 1A, HNE–protein adducts were detected in lavaged lung cells following ozone exposure. Control cells have endogenous HNE–protein adducts, most likely as a result of basal formation of intracellular free radicals. Immediately following cessation of ozone exposure there was a visible increase in HNE adducts associated with a protein at approximately 32 kDa and a second one at 86–90 kDa. The intensity of HNE adduct formation continues to increase with time and peaks at approximately 3 hr. Some adduct is seen at lower molecular weight and migrates with the dye front; it could be degradation products of HNE adducts or very-low-molecular-weight adducts. Consequently, there appears to be some specificity in the formation and/or intensification of two protein adducts in lung cells following ozone exposure. The bands on the Westerns corresponding to the 32-kDa adduct from the replicate experiments were scanned and the results are presented in Fig. 1B. Similar to the results in Fig. 1A, the 32-kDa HNE–protein adduct increases following ozone exposure until approximately 3 hr postexposure.

In order to determine whether HNE adducts could be detected at lower ozone exposures, mice were exposed to 0.25 ppm ozone for 3 hr. Mice were euthanized 3 hr after cessation of ozone exposure and HNE adducts examined in alveolar macrophages by Western analysis. As can be seen in Fig. 2, an increase in HNE adducts was evident following ozone exposure including a protein at 32 kDa. Under these conditions, there was no evidence of inflammation. Consequently, even relatively low ozone exposures cause the formation of HNE adducts.

Ozone-Induced Lung Cell Injury

The time-dependent morphological changes that occur in the lung lavage cells after ozone exposure were also exam-

PM3006739320

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

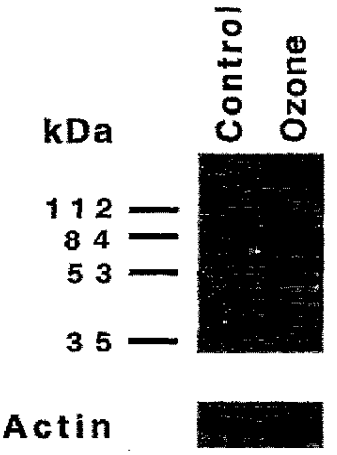

FIG. 2. HNE–protein adducts in murine lung cells following *in vivo* exposure to 0.25 ppm ozone for 3 hr. HNE adducts were detected in lung cells lavaged 3 hr after ozone exposure by Western analysis, similar to Fig. 1. Each lane contains pooled cells from three C3H/HeJ mice. Actin levels, detected by Western analysis are shown to demonstrate that the increase in the HNE adducts following ozone are not due to differences in cell loading.

ined. Cytoplasmic degeneration and cell lysis were observed in AM after exposing mice to ozone for 3 hr at 2.0 ppm. Similar to other reports (Gilmour *et al.*, 1993a; Gilmour and Selgrade, 1993) ozone exposure also resulted in polymorphonuclear neutrophil (PMN) influx that was detectable 1.5 hr after ozone exposure and peaked at 6 hr (Fig. 3). Desquamated epithelial cells were occasionally found in the lavage at 3 and 6 hr following ozone exposure (data not shown). The morphologic evidence of necrosis correlated with trypan blue exclusion data showing the maximum presence of necrotic cells from lavage at 1.5, 3, and 6 hr following ozone exposure (Fig. 4).

Ozone-Induced Apoptosis of Alveolar Macrophages

Although there was evidence of cellular necrosis following 2.0 ppm ozone exposure for 3 hr, we investigated whether cellular apoptosis was also occurring due to ozone-induced oxidative stress. Cells recovered from lung lavage (primarily alveolar macrophages) were undergoing apoptosis with typical nuclear condensation and fragmentation, cell shrinkage, and cytoplasmic condensation (Fig. 5). In order to confirm that the morphologic changes were due to apoptosis, Cell Death ELISA and DNA fragmentation assays were performed. The DNA of lung cells from ozone-treated mice had significant amounts of fragmentation at 6 hr after exposure as shown by DNA ladder formation (Fig. 6), consistent with apoptotic cell injury. It can also be seen from the Cell Death ELISA results that there was a significant increase in apoptosis at 3 and 6 hr following ozone exposure, as indicated by increases in cytosolic histone-bound DNA fragments in these cells (Fig. 7).

Since PMN undergo rapid apoptosis (Blackwell and Crawford, 1992), it was not certain whether ozone was causing apoptosis or whether the apoptosis was secondary to the PMN influx. Therefore, lung lavage cells from mice obtained immediately after cessation of ozone exposure (95% alveolar macrophages) were cultured for 3 hr at 1×10^6 cells/ml in 1 ml Hepes-buffered Medium-199 (Gibco BRL) with 10% heat-activated fetal bovine serum (FCS, Sigma) and analyzed for evidence of apoptosis (Figs. 8 and 9). Since cells obtained at this time point contained no PMN (Fig. 3) the morphological appearance of apoptosis (Fig. 8) was due to ozone and not the PMN influx. Consistent with these results, it can be seen that there was a significant increase in apoptosis, detected by Cell Death ELISA, after the 3 hr of incubation following ozone exposure (Fig. 9).

DISCUSSION

This study demonstrates that exposure of C3H/HeJ mice to ozone resulted in the formation of HNE-modified proteins in lung cells. Following ozone exposure, two cellular proteins at approximately 32 and 86–90 kDa were detected with increased HNE adducts. The formation of two primary

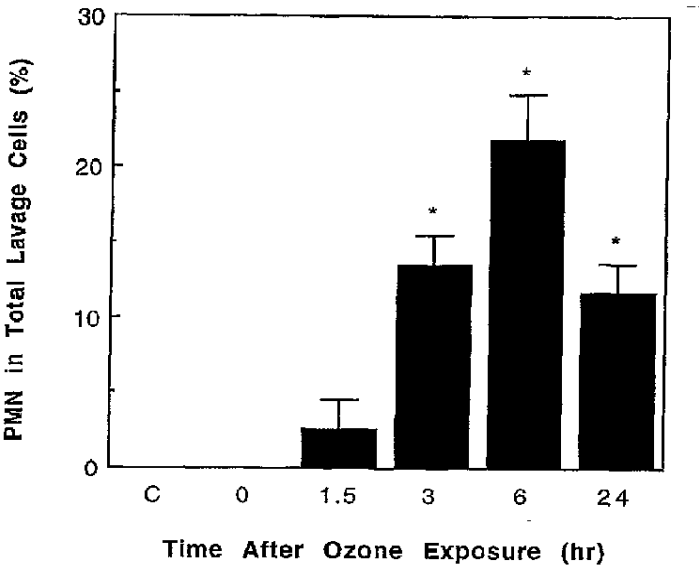

FIG. 3. Ozone-induced PMN influx as a function of time following 2.0 ppm ozone exposure for 3 hr. At each of the time points indicated the lung lavage cells from three mice were pooled and the number of PMN in each sample was determined by microscopic examination as described under Materials and Methods. There was a significant increase of PMN following exposure to 2.0 ppm ozone. *Significant at $p \leq 0.05$ compared with the control group ($n = 3$).

PM3006739321

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

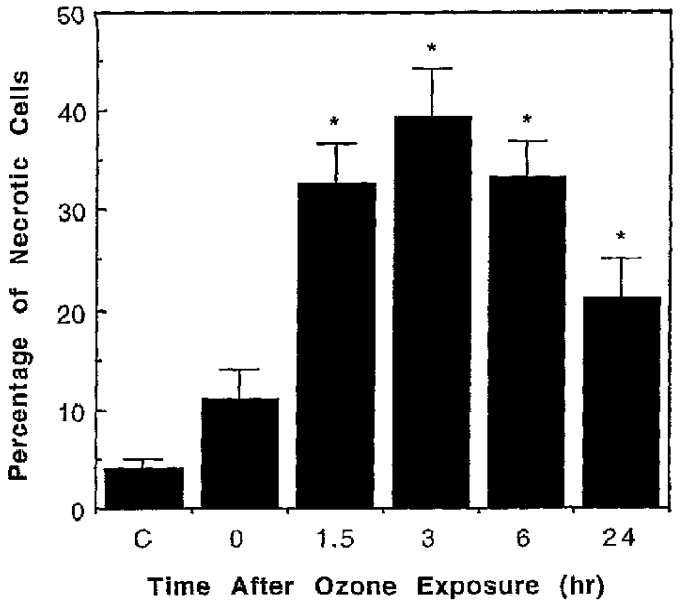

FIG. 4. Ozone-induced decrease in lung lavage cell viability following 3 hr 2.0 ppm ozone exposure. Cell viability was determined for a pool of cells from three mice, as soon as possible following lung lavage at each of the time points indicated, by trypan blue exclusion as described under Materials and Methods. The data indicate an increase in necrotic cells in lung lavage following 2.0 ppm ozone exposure. *Significant at $p \leq 0.05$ compared with the control group ($n = 3$).

adducts implies some specificity in the formation of these adducts. The mechanism and significance of this apparent specificity are uncertain, but may have to do with the function of the proteins, the number of exposed cysteine residues, and/or the intracellular location of the proteins. One potential candidate is an important intracellular antioxidant protein, heme oxygenase-1, that is also known as the 32-kDa stress protein (Keyse and Tyrrell, 1987). Whether heme oxygenase-1 serves as a target for HNE remains to be determined. A number of other stress proteins are in the range of 86–90 kDa and could account for the other primary HNE–protein adduct.

The presence of HNE–protein adducts immediately after ozone exposure and in the absence of any inflammation (e.g., 0.25 ppm ozone) supports the hypothesis that HNE was formed as a result of lipid peroxidation of PUFA following ozone exposure. Whether the source of the PUFA is within the lipids of the lung lining layer or at the cell membrane cannot be determined from these studies. The hypothesis that diffusible toxic products such as HNE can originate from the peroxidative breakdown of PUFA is supported by several lines of research (Esterbauer *et al.*, 1986, 1991).

The amount of HNE-modified proteins increased following cessation of ozone exposure and reached a peak at 3 hr for the 86- to 90-kDa protein, but continued to increase for the 32-kDa protein up to the last time point examined (24 hr). Since HNE may take some time to react with cellular proteins, this could contribute to the observed time course. HNE may also continue to be formed following cessation of ozone exposure by continued peroxidation and sustained PUFA breakdown, or contribution from inflammatory PMN. Our data indicated a PMN influx in bronchoalveolar lavage by 3 and 6 hr after ozone exposure. Since HNE in the range of 0.1–1 μM has been shown to be chemotactic for human and murine PMN leukocytes (Curzio *et al.*, 1990; Schaur *et al.*, 1991), it is possible that HNE formed initially during

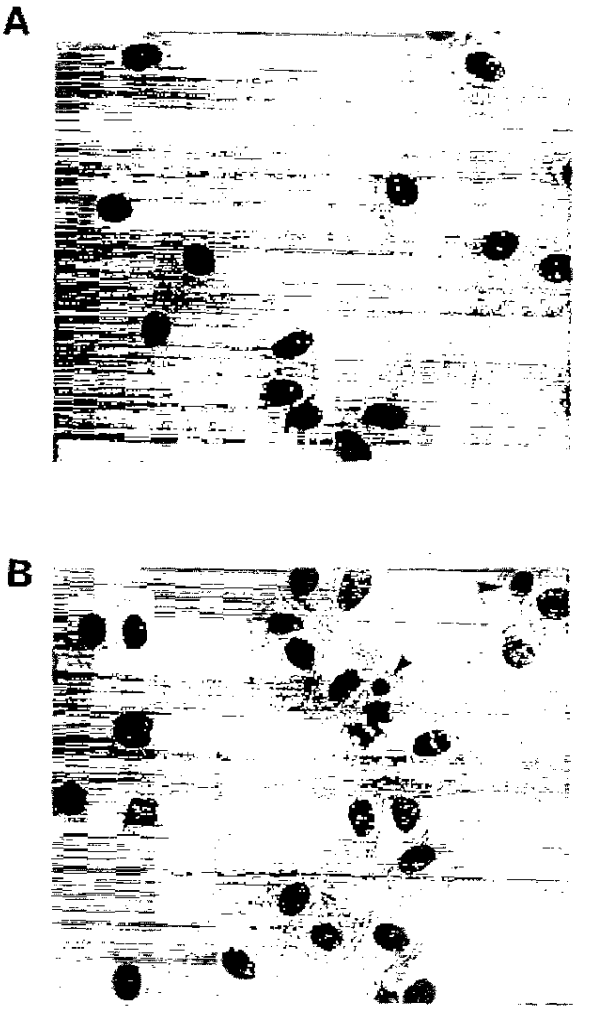

FIG. 5. Morphologic evidence of alveolar macrophage apoptosis following ozone exposure. Representative photomicrographs of alveolar macrophages 3 hr following air (A) and ozone exposure to 2.0 ppm for 3 hr (B) stained with Wright Giemsa as described under Materials and Methods. Cytoplasmic and nuclear degeneration was observed in alveolar macrophages from C3H/HeJ mice following exposure to ozone. Some cells (marked) appear apoptotic with nuclear and cytoplasmic condensation and cell shrinkage. Original magnification is 630× (dry) in bright field.

PM3006739322

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

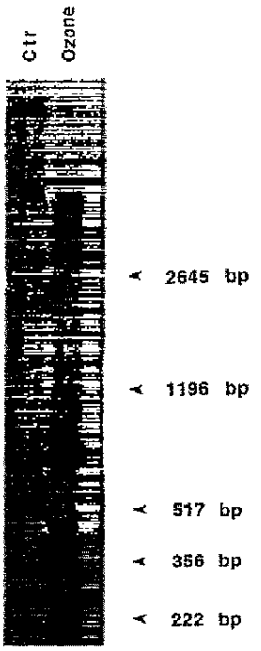

FIG. 6. DNA "ladder" formation in lung cells following ozone exposure. DNA was isolated from pooled (eight mice for each lane) lung lavage cells recovered from mice 6 hr after exposure to air (ctr) or 2.0 ppm ozone for 3 hr and treated as described under Materials and Methods. A DNA ladder is evident in the lane containing cells obtained from ozone-exposed mice. The results demonstrate that DNA fragmentation occurred following ozone exposure, consistent with apoptotic changes in cells.

ozone exposure would be sufficient to induce or contribute to the PMN influx. Furthermore, it has been reported that neutrophils contribute to the process of HNE formation during acute inflammation and are associated with the production of superoxide anion (Schaur *et al.*, 1994). Therefore, it is possible that increased numbers of PMN recruited to the lung in ozone exposed mice release an increased amount of oxy-radicals that are able to initiate additional lipid peroxidation and HNE formation, which may explain the continued increase in the 32-kDa protein adduct. However, the result from the 0.25 ppm ozone exposure is consistent with the hypothesis that HNE can be formed in the absence of measurable inflammation.

Although 3 hr of 2.0 ppm ozone exposure causes cell injury by a necrotic mechanism, this study indicated that ozone can also cause lung cell injury by an apoptotic mechanism. Apoptosis was evident from morphological examination of cells and was consistent with results obtained by the Cell Death assay and by DNA ladder formation. Consistent with this observation, HNE has been shown to induce macrophage apoptosis *in vitro* at ≤ 100 μM concentrations, while higher concentrations result in cellular necrosis (Li *et al.*, 1996). Since HNE was formed during ozone exposure it may account or contribute to the oxidative lung injury observed in this model. Since the mechanism by which HNE causes alveolar macrophages injury is dose dependent, it would suggest that low ozone concentrations would cause apoptosis in the absence of necrosis. In preliminary results, using a murine model, ozone exposures of 0.4 ppm for 3 hr caused apoptosis in the absence of necrosis (data not shown).

Lung lavage cells obtained immediately after cessation of ozone exposure (that contained no PMN) also showed signs of apoptosis when cultured for 3 hr. The extent of apoptosis (see Figs. 7 and 9) was less in the cultured cells compared to cells isolated 3 hr postexposure. This may in part be explained by the fact that the cells have been exposed to less HNE since fewer adducts are evident (see Fig. 1). It is also possible that some protection may be provided by the culture medium. Nevertheless, these results support the hypothesis that apoptosis was induced by ozone exposure, required time for the apoptotic process to occur, and was not a result of neutrophil infiltration alone.

Since other toxic aldehydes are also formed during ozone exposure (Cueto *et al.*, 1992; Pryor and Church, 1991; Pryor *et al.*, 1991a,b; Rabinowitz and Basset, 1988), it is possible that HNE contributes to, rather than is solely responsible

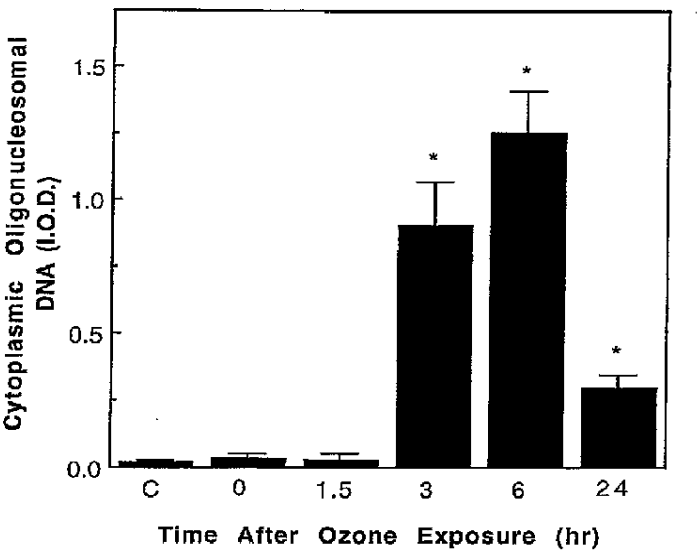

FIG. 7. Detection of cytosolic histone-bound DNA fragments (indicating apoptosis) in lung lavage cells following ozone exposure. Lung lavage cells were pooled from three mice, similar to Fig. 1, and examined for cytosolic histone-bound DNA fragments using a Cell Death ELISA assay as described under Materials and Methods. The results are expressed as O.D. There were significant increases in apoptosis 3 and 6 hr following ozone exposure as indicated by increases in cytosolic histone-bound DNA fragments in these cells. *Significant at $p \leq 0.05$ compared with the control group ($n = 3$).

PM3006739323

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

for, the cell injury. Consequently, it may be the combination of these reactive metabolites that causes lung injury, with HNE contributing significantly due to its high toxicity.

This study primarily utilized a 2.0 ppm ozone exposure for 3 hr. Although this exposure condition does not reflect typical human ozone exposure, it can be used as a model of oxidative stress upon which to establish potential mechanisms of oxidative lung injury. Furthermore, since 0.25 ppm ozone exposure also caused formation of HNE adducts in alveolar macrophages it suggests that HNE can be formed over a wide range of exposures. Consistent with these observations, Hamilton *et al.* (1996) have recently reported that human subjects exposed to 0.25 ppm for 1 hr also had an increase in HNE adducts on alveolar macrophages and were also primarily on a 32-kDa protein.

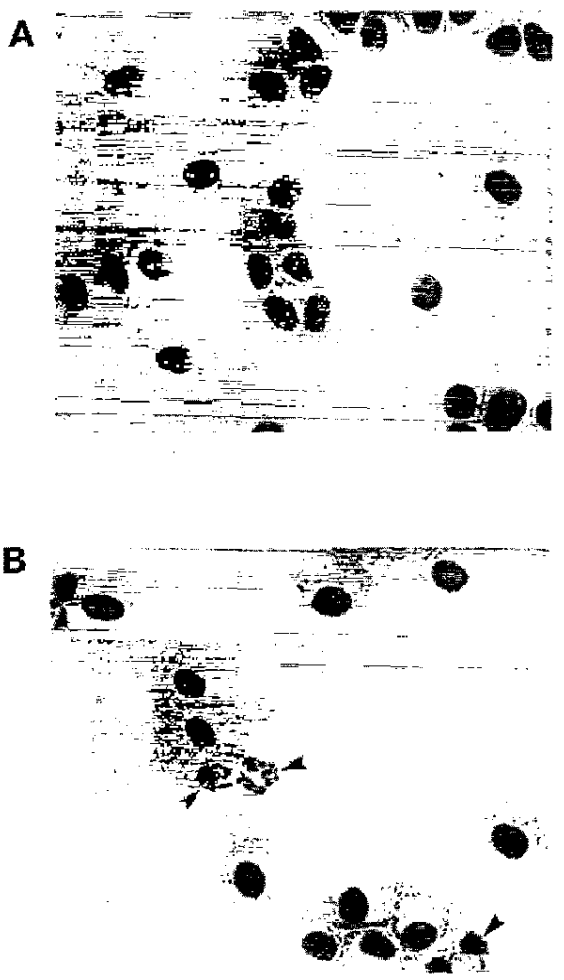

FIG. 8. Photomicrographs of alveolar macrophages recovered immediately after cessation of air (A) or 2.0 ppm ozone for 3 hr (B) and cultured for an additional 3 hr. Cells were examined in a similar manner as described in the legend to Fig. 5. Nuclear condensation and cell shrinkage (marked) were observed in AM after ozone exposure. Original magnification is 630× (dry) in bright field.

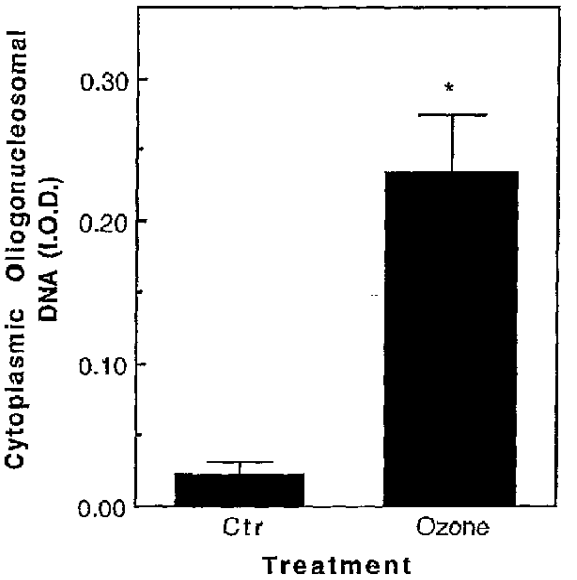

FIG. 9. Detection of cytosolic histone-bound DNA fragments in lung lavage cells recovered immediately after ozone exposure and cultured for 3 hr. Cell Death ELISA was used to quantitate cytosolic histone bound DNA fragments as described in the legend to Fig. 7 on cells cultured as described in the legend to Fig. 8. The results indicate a significant increase in apoptosis in cultured cells obtained from ozone-exposed mice compared to control. *Significant at $p \leq 0.05$ compared with the control group ($n = 3$).

In summary, these results demonstrate that ozone *in vivo* induces the formation of HNE. In turn, formation of HNE adducts with proteins that may be associated with apoptosis and necrosis reflects the relative pathogenic importance of HNE as a secondary toxic messenger for acute ozone injury. The formation of highly toxic intermediates such as HNE should provide insights for mechanisms to better understand ozone-mediated cell injury. The observation that ozone causes apoptosis of lung cells describes an additional type of cell injury that can occur following ozone exposure.

REFERENCES

Bhalla, D. K., Mannix, R. C., Kleinman, M. T., and Crocker, T. T. (1986). Relative permeability of nasal, tracheal, and bronchoalveolar mucosa to macromolecules in rats exposed to ozone. *J. Toxicol. Environ. Health* **17**, 269–283.

Blackwell, S., and Crawford, J. (1992). Colony-stimulating factors: Clinical applications. *Pharmacotherapy* **12**, 20S–31S.

Buttke, T. M., and Sandstrom, P. A. (1994). Oxidative stress as a mediator of apoptosis [see comments]. *Immunol. Today* **15**, 7–10.

Chang, L. Y., Huang, Y., Stockstill, B. L., Graham, J. A., Grose, E. C., Menache, M. G., Miller, F. J., Costa, D. L., and Crapo, J. D. (1992). Epithelial injury and interstitial fibrosis in the proximal alveolar regions

PM3006739324

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

of rats chronically exposed to a simulated pattern of urban ambient ozone. *Toxicol. Appl. Pharmacol.* **115,** 241–252.

Cueto, R., Squandrito, G. L., Bermudez, E., and Pryor, W. (1992). Identification of heptanal and nonenal in brochoalveolar lavage from rats exposed to low levels of ozone. *Biochem. Biophys. Res. Commun.* **188,** 129–134.

Curzio, M., Esterbauer, H., Mauro, C. D., and Dianzani, M. U. (1990). Influence of the lipid peroxidation product 4-hydroxynonenal on human neutrophil migration. *Int. J. Tissue React.* **6,** 13–18.

Danielson, U. H., Esterbauer, H., and Mannervik, B. (1987). Structure-activity relationships of 4-hydroxyalkenals in the conjugation catalysed by mammalian glutathione transferases. *Biochem. J.* **247,** 707–713.

Devlin, R. B., McDonnell, W. F., Mann, R., Becker, S., House, D. E., Schreinemachers, D., and Koren, H. S. (1991). Exposure of humans to ambient levels of ozone for 6.6 hours causes cellular and biochemical changes in the lung. *Am. J. Respir. Cell Mol. Biol.* **4,** 72–81.

Dianzani, M. U., Paradisi, L., Barrera, G., Rossi, M. A., and Parola, M. (1989). The action of 4-hydroxynonenal on the plasma membrane enzymes from rat hepatocytes. In *Free Radicals, Metal Ions and Biopolymers* (P. C. Beaumont, D. J. Deeble, B. J. Parsons, and C. Rice-Evans, Eds.), pp. 329–346. Richelieu Press, London.

Driscoll, K. E., Vollmuth, T. A., and Schlesinger, R. B. (1987). Acute and subchronic ozone inhalation in the rabbit: Response of alveolar macrophages. *J. Toxicol. Environ. Health* **21,** 27–43.

Esterbauer, H., Benedetti, A., Lang, J., Fulceri, R., Fauler, G., and Comportiz, M. (1986). Studies on the mechanism of formation of 4-hydroxynonenal during microsomal lipid peroxidation. *Biochim. Biophys. Acta.* **876,** 154–166.

Esterbauer, H., Schaur, R. J., and Zollner, H. (1991). Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Rad. Biol. Med.* **11,** 81–128.

Gilmour, M. I., Hmieleski, R. R., Stafford, E. A., and Jakab, G. J. (1991). Suppression and recovery of the alveolar macrophage phagocytic system during continuous exposure to 0.5 ppm ozone. *Exp. Lung Res.* **17,** 547–548.

Gilmour, M. I., Park, P., Doerfler, D., and Selgrade, M. K. (1993a). Factors that influence the suppression of pulmonary antibacterial defenses in mice exposed to ozone. *Exp. Lung Res.* **19,** 299–314.

Gilmour, M. I., Park, P., and Selgrade, M. K. (1993b). Ozone-enhanced pulmonary infection with Streptococcus zooepidemicus in mice. The role of alveolar macrophage function and capsular virulence factors. *Am. Rev. Respir. Dis.* **147,** 753–760.

Gilmour, M. I., and Selgrade, M. K. (1993). A comparison of the pulmonary defenses against streptococcal infection in rats and mice following O_3 exposure: Differences in disease susceptibility and neutrophil recruitment. *Toxicol. Appl. Pharmacol.* **123,** 211–218.

Hamilton, R. F., Hazbun, M. E., Jumper, C. A., Eschenbacher, W. L., and Holian, A. (1996). 4-Hydroxynonenal mimics ozone-induced modulation of macrophage function ex vivo. *Am. J. Respir. Cell Mol. Biol.* **15,** 275–282.

Harkema, J. R., Plopper, C. G., Hyde, D. N., George, J. A. S., and Dungworth, D. L. (1987). Response of the macaque epithelium to ambient levels of ozone. *Am. J. Pathol.* **128,** 29–32.

Hotchkiss, J. A., Harkema, J. R., Kirkpatrick, D. T., and Henderson, R. F. (1989). Response of rat alveolar macrophages to ozone: Quantitative assessment of population size, morphology, and proliferation following acute exposure. *Exp. Lung Res.* **15,** 1–16.

Jakab, G. J., and Hemenway, D. R. (1994). Concomitant exposure to carbon black particulates enhances ozone-induced lung inflammation and suppression of alveolar macrophage phagocytosis. *J. Toxicol. Environ. Health* **41,** 221–231.

Keyse, S. M., and Tyrrell, R. M. (1987). Both near ultraviolet radiation and the oxidizing agent hydrogen peroxide induce a 32-kDa stress protein in normal human skin fibroblasts. *J. Biol. Chem.* **262,** 14821–14825.

Koren, H. S., Devlin, R. B., Graham, D. E., Mann, R., McGee, M. P., and Horstman, D. H. (1989). Ozone-induced inflammation in the lower airways of human subjects. *Am. Rev. Respir. Dis.* **139,** 407–415.

Last, J. A., Gelzleichter, T. R., Pinkerton, K. E., Walker, R. M., and Witschi, H. (1993). A new model of progressive pulmonary fibrosis in rats. *Am. Rev. Respir. Dis.* **148,** 487–494.

Li, L., Hamilton, R. F., Jr., Kirichenko, A., and Holian, A. (1996). 4-Hydroxynonenal-induced cell death in murine alveolar macrophages. *Toxicol. Appl. Pharmacol.* **139,** 135–143.

Loveren, H. V., Rombout, P. J., Wagenaar, S. S., Walvoort, H. C., and Vos, J. G. (1988). Effects of ozone on the defense to a respiratory *Listeria monocytogenes* infection in the rat: Suppression of macrophage function and cellular immunity and aggravation of histopathology in lung and liver during infection. *Toxicol. Appl. Pharmacol.* **94,** 374–393.

Michiels, C., and Remacle, J. (1991). Cytotoxicity of linoleic acid peroxide, malondialdehyde and 4-hydroxynonenal towards human fibrosis. *Toxicology* **66,** 225–234.

Pendino, K. J., Shuler, R. L., Laskin, J. D., and Laskin, D. L. (1994). Enhanced production of interleukin-1, tumor necrosis factor-alpha, and fibronectin by rat lung phagocytes following inhalation of a pulmonary irritant. *Am. J. Respir. Cell Mol. Biol.* **11,** 279–286.

Polzer, G., Lind, I., Mosbach, M., Schmidt, A., and Seidel, A. (1994). Combined influence of quartz dust, ozone and NO_2 on chemotactic mobility, release of chemotactic factors and other cytokines by macrophages in vitro. *Toxicol. Lett.* **72,** 307–315.

Pryor, W. A. (1986). Oxy-radicals and related species: Their formation, lifetimes and reactions. *Annu. Rev. Physiol.* **48,** 657–667.

Pryor, W. A., and Church, D. F. (1991). The reaction of ozone with unsaturated fatty acids: Aldehydes and hydrogen peroxide as mediators of ozone toxicity. In *Oxidative Damage and Repair: Chemical, Biological and Medical Aspects* (K. J. A. Davies, Ed.), pp. 496–504. Pergamon Press, New York.

Pryor, W. A., Das, B., and Church, D. F. (1991a). The ozonation of unsaturated fatty acids; Aldehydes and hydrogen peroxide as products and possible mediators of ozone toxicity. *Chem. Res. Toxicol.* **4,** 341–348.

Pryor, W. A., Miki, M., Das, B., and Church, D. F. (1991b). The mixture of aldehydes and hydrogen peroxide produced in the ozonation of dioleoyl phosphatidylcholine causes hemolysis of human red blood cells. *Chem. Biol. Interact.* **79,** 41–52.

Rabinowitz, J. L., and Basset, D. J. P. (1988). Effect of 2 ppm ozone exposure on rat lung lipid fatty acids. *Exp. Lung Res.* **14,** 477–489.

Reiser, K. M., Tyler, W. S., Hennessy, S. M., Dominguez, J. J., and Last, J. A. (1987). Long-term consequences of exposure to ozone. II. Structural alterations in lung collagen of monkeys. *Toxicol. Appl. Pharmacol.* **89,** 314–322.

Rossi, M. A., Fidale, F., Garramone, A., Esterbauer, H., and Dianzani, M. U. (1990). Effect of 4-hydroxyalkenals on hepatic phosphatidylinositol-4,5-biphosphate-phospholipase C. *Biochem. Pharmacol.* **39,** 1715–1719.

Schaur, R. J., Dusseng, G., Kink, E., Schaurenstein, E., Posch, W., Kukovetz, E., and Egger, G. (1994). The lipid peroxidation product 4-hydroxynonenal is formed by—and is able to attract—rat neutrophils in vivo. *Free Radical Res.* **20,** 365–373.

Schaur, R. J., Zollner, H., and Esterbauer, H. (1991). Biological effects of aldehydes with particular attention to 4-hydroxynonenal and malonaldehyde. In *Membrane Lipid Oxidation* (C. Vigo-Pelfrey, Ed.), pp. 141–163. CRC Press, Boca Raton.

PM3006739325

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001

Szweda, L. I., Uchida, K., Tsai, L., and Stadtman, E. R. (1993). Inactivation of glucose-6-phosphate dehydrogenase by 4-hydroxy-2-nonenal. *J. Biol. Chem.* **268,** 3342–3347.

Taher, M. M. (1993). 4-Hydroxynonenal, a metabolite of lipid peroxidation, activates phospholipase D in vascular endothelial cells. *Free Radical Biol. Med.* **15,** 365–375.

Toyokuni, S., Uchida, K., Okamoto, K., Hattori-Nakakuki, Y., Hiai, H., and Stadtman, E. R. (1994). Formation of 4-hydroxy-2-nonenal-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate. *Proc. Natl. Acad. Sci. USA* **91,** 2616–2620.

Uchida, K., and Stadtman, E. R. (1993). Covalent attachment of 4-hydroxynonenal to glyceraldehyde-3-phosphate dehydrogenase. *J. Biol. Chem.* **268,** 6388–6393.

Uchida, K., Szweda, L., Chae, H., and Stadtman, E. (1993). Immunochemical detection of 4-hydroxynonenal protein adducts in oxidized hepatocytes. *Proc. Natl. Acad. Sci. USA* **90,** 8742–8746.

VanWinkle, W. B., Snuggs, M., and Buja, L. M. (1994). Cytoskeletal alterations in cultured cardiomyocytes following exposure to the lipid peroxidation product, 4-hydroxynonenal. *Cell. Motil. Cytoskeleton* **28,** 119–134.

Victor, K. (1992). Review of the genotoxicity of ozone. *Mutat. Res.* **277,** 221–238.

Vincent, R., Chang, L. Y., Mercer, R. R., Miller, F. J., and Crapo, J. D. (1989). Interstitial pulmonary fibrosis in rodents following chronic exposure to an ambient pattern of ozone. *Am. Rev. Respir. Dis.* **139,** A278.

Wilson, D. W., Plopper, C. G., and Dungworth, D. L. (1984). The response of macaque tracheobronchial epithelium to acute ozone injury. *Am. J. Pathol.* **116,** 193–206.

Wright, E. S., Dziedzic, D., and Wheeler, C. S. (1990). Cellular, biochemical and functional effects of ozone: New research and perspectives on ozone health effects. *Toxicol. Lett.* **51,** 125–145.

Zollner, H., Schaur, R. J., and Esterbauer, H. (1991). Biological activities of 4-hydroxyalkenals. In *Oxidative Stress: Oxidants and Antioxidants* (H. Sies, Ed.), pp. 337–369. Academic Press, New York.

PM3006739326

Source: https://www.industrydocuments.ucsf.edu/docs/ftnx0001